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Evaluation of Two *Salmonella typhimurium* Hybrids as Challenge Organisms in a System for the Assay of Typhoid Vaccines

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A mouse-virulent *Salmonella typhimurium* hybrid (H42), which expresses the *Salmonella typhi* Vi antigen in addition to *S. typhi* O antigens 9 and 12, and a mouse-virulent *S. typhimurium* hybrid (H1), which expresses only the 9 and 12 antigens of *S. typhi*, were compared in their behavior as challenge organisms in a system developed to assay the protective capabilities of typhoid vaccines. Swiss-Webster white mice, vaccinated intraperitoneally with live *Escherichia coli* hybrids expressing the *S. typhi* O antigens 9 and 12, were significantly protected against death from intraperitoneal challenge with each of the *S. typhimurium* hybrid strains. Vaccination with an *E. coli* hybrid expressing the *S. typhi* Vi antigen in addition to O antigens 9 and 12 was seen to confer no advantage in protection against either *S. typhimurium* hybrid challenge organism over that obtained by vaccination with an *E. coli* hybrid expressing only the O antigens of *S. typhi*. However, a notable difference in the behavior of the two *S. typhimurium* hybrids was seen in mice vaccinated with the parent of the *E. coli* hybrid vaccinating strains, *E. coli* F464, which expresses no surface antigens common to either of these *S. typhimurium* hybrid challenge organisms. A nonspecific (with respect to the vaccinating strain) protective effect, believed to be associated with Vi antigen expression by the challenge organism, was seen against the challenge with *S. typhimurium* hybrid H42 after F464 vaccination, whereas no protection was conferred by F464 vaccination against the challenge with Vi-nonexpressing *S. typhimurium* hybrid H1. Inasmuch as neither *S. typhimurium* hybrid discriminates between the expression or nonexpression of the Vi antigen in a vaccinating strain, it is concluded that the Vi-nonexpressing *S. typhimurium* hybrid H1, which more clearly indicates the vaccine-specific protective role of the *S. typhi* O antigens and does not exhibit the nonspecific protection response of hybrid H42, is the better choice as challenge organism for this vaccine assay system.

In previous studies (1-5) aimed at developing an animal protection system suitable for the assay of vaccines against typhoid fever in humans, we have examined the feasibility of using mouse-virulent *Salmonella typhimurium* hybrids expressing *Salmonella typhi* surface antigens as challenge organisms in vaccinated Swiss-Webster white mice. Our initial expectation was that the ideal hybrid for this purpose would be one expressing a complete set of these *S. typhi*-derived antigens, i.e., the O antigens 9 and 12, the flagellar antigen d, and the Vi antigen. Such a hybrid is *S. typhimurium* H42, and, indeed, this strain has been employed as a challenge organism in every one of our previous studies with this assay system (1-5). However, several factors have become apparent during the course of these studies which suggest that an *S. typhimurium* hybrid expressing only the O antigens of *S. typhi* might be more suitable for this purpose than hybrid H42.

In an earlier study (1), we conducted experiments in which *S. typhimurium* hybrids, expressing different combinations of *S. typhi*-derived surface antigens, were used as challenge organisms in mice immunized with nonliving vaccines prepared from strains of *S. typhimurium*, *S. typhi*, and *S. typhimurium* hybrids that expressed the *S. typhi* surface antigens; the results of these experiments indicated that the immune response generated against the O surface antigens of the challenge organism is an important factor in determining the ability of the vaccine to protect the animals against death.

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This importance of the O antigen antibodies in the protection of mice against *Salmonella* infection was suggested earlier by the studies of Ornellas et al. (13) and has subsequently been confirmed by the work of Sevenson et al. (16) as well as that of Lyman et al. (12). However, our studies with these *S. typhimurium* hybrids have also indicated that only those immunogens directed against the O antigens of the challenge organism are involved in the vaccine-specific protection conferred in this system; no apparent protective role has been observed for immunogens against either the Vi antigen or the flagellar antigens expressed by the challenge organism (1, 3–5). Furthermore, one of our earliest studies suggested that expression of the Vi antigen by an *S. typhimurium* hybrid may alter its virulence characteristics in a manner which adversely affects its ability to respond properly as a challenge organism, with the result that some vaccines protect nonspecifically against such hybrids (1). An especially disturbing example of this nonspecific protective effect was seen in our most recent study (3), in which mice vaccinated with the *Escherichia coli* strain F464, which expresses no surface antigens common to *S. typhimurium* hybrid H42, were significantly protected against death after challenge with that hybrid. In view of these findings, a reconsideration of the merit of using hybrid H42 as a challenge organism in this system seemed in order. In the present communication, we compare the behavior of *S. typhimurium* hybrid H42 as a challenge organism with that of *S. typhimurium* hybrid H1, which expresses only the O surface antigens of *S. typhi*, and we argue that hybrid H1 is the better choice for vaccine assay.

MATERIALS AND METHODS

Bacterial strains. The male parent of all of the hybrids employed in this study is *S. typhi* WR4000 (serotype O9, 12; Vi; d), originally designated TD-7, an Hfr strain whose derivation and description were reported previously (8). Kiefer et al. (10) described the generation of *E. coli* hybrid F1061 from the mating between *S. typhi* WR4000 and *E. coli* F464 (serotype O8; K$^-$; H$^-$) as the consequence of recombination at the *rfb* loci. *E. coli* hybrid F1061 expresses the *S. typhi* O antigens 9 and 12 and lacks the O8 specificity of its *E. coli* parent F464. *E. coli* hybrid WR3078 was derived from *E. coli* hybrid F1061 by transfer of the Vi antigen-determining (*viaB*$^+$) genes from *S. typhi* WR4000 (3); *E. coli* hybrid WR3078 expresses the *S. typhi* Vi antigen in addition to *S. typhi* O antigens 9 and 12. The *S. typhimurium* hybrid challenge organisms H1 and H42, originally referred to as WR5004 His$^+$, 9, 12; i-1, 2 and WR5004 His$^+$, Ara$^+$-2, 9, 12, Vi; d-1, 2, respectively, were derived from the mouse-virulent *S. typhimurium* strain WR5004 by mating with *S. typhi* WR4000, as described in a previous communication (2). *S. typhimurium* hybrid H42 expresses the *S. typhi* O antigens 9 and 12, the *S. typhi* Vi antigen, and the *S. typhi* flagellar antigen d, whereas *S. typhimurium* hybrid H1 expresses only the 9 and 12 O antigens of *S. typhi*; each of these hybrids has a 50% mouse lethal dose of less than 50 organisms. The well-known *S. typhi* Ty2 strain (O9, 12; Vi; d) has been employed in our previous protection studies (1–5), and the *S. typhi* Ty2W strain is a Vi-nonexpressing derivative of Ty2.

Protection experiments. Organisms were harvested in phosphate-buffered saline, washed three times, and appropriately diluted. Swiss-Webster white mice (40 per group) HPB strain, random bred, and weighing 16 to 18 g were vaccinated intraperitoneally with 10^7 live organisms of the immunizing strain suspended in 0.5 ml of solution. (In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.) The animals tolerated this vaccinating dose well with all of the immunizing strains employed, and deaths resulting from toxemia seldom occurred. Mice were challenged 5 weeks later with 2,500 organisms (in 0.5 ml) of *S. typhimurium* hybrid H42 or *S. typhimurium* hybrid H1 administered intraperitoneally. Survivors were counted 21 days after challenge.

Test for statistical significance. The results of the assays were analyzed as RXC tables by using the usual chi-square criterion (15).

RESULTS

S. typhimurium hybrid H42, expressing the *S. typhi* surface antigens 9, 12, d, and Vi, has been used previously as a challenge organism in testing the live vaccinating strains *E. coli* hybrid F1061, *E. coli* hybrid WR3078, and their parent, *E. coli* F464 (3); its response to those strains in the present study was similar to that which we observed in the earlier study. *S. typhimurium* hybrid H1, which expresses only the 9 and 12 O surface antigens of *S. typhi*, has not been employed previously as a challenge organism with any of the live vaccinating strains examined here. The comparison of the behavior of hybrid H1 with that of hybrid H42, as challenge organisms in protection experiments with five live vaccinating strains, is shown in Table 1.

The notable difference in the behavior of the two *S. typhimurium* hybrid challenge organisms was seen in those mice vaccinated with the live *E. coli* strain F464. The nonspecific (with respect to the vaccinating strain) protective effect against hybrid H42 resulted in the survival of 50% of the F464-vaccinated animals challenged with this hybrid, which is a significant level of protection ($P < 0.005$). In contrast, similarly F464-vaccinated mice were not protected when *S. typhimurium* hybrid H1 was employed as the challenge organism.

E. coli hybrids expressing *S. typhi* O antigen specificity have been suggested as possible candidates for use in human subjects to develop a

TABLE 1. Comparison of the behavior of *S. typhimurium* hybrids H1 and H42 as challenge organisms in intraperitoneally vaccinated mice[a]

Vaccinated with[b]:	Challenged with[b]: *S. typhimurium* H42 (O9, 12: Vi:d: 1, 2)		*S. typhimurium* H1 (O9, 12:i:1, 2)	
	Survivors/no. inoculated	% Survival	Survivors/no. inoculated	% Survival
S. typhi Ty2 (O9, 12, Vi:d)	37/40[c]	92.5	34/40[c]	85.0
S. typhi Ty2W (O9, 12: d)	36/40[c]	90.0	32/40[c]	80.0
E. coli F1061 (O9, 12)	29/40[c]	72.5	22/40[c]	55.0
E. coli WR3078 (O9, 12: Vi)	28/40[c]	70.0	20/40[c]	50.0
E. coli F464 (O8)	20/40[c]	50.0	4/40	10.0
Control (not vaccinated)	2/40	5.0	1/40	2.5

[a] The vaccinating dose was 10^7 live organisms, and the animals were challenged intraperitoneally after 5 weeks with 2,500 organisms of either *S. typhimurium* hybrid H1 or *S. typhimurium* hybrid H42.

[b] Surface antigens expressed by the vaccinating strains and the challenge strains are shown in parentheses.

[c] Mortality is significantly less ($P < 0.005$) than in the control group.

safe, orally administered, vaccine for the prevention of typhoid fever (10). Vaccination here with either of the F464-derived live *E. coli* hybrids F1061 (expressing the *S. typhi* O antigens 9 and 12) or WR3078 (expressing the *S. typhi* O antigens 9 and 12 and the Vi antigen) conferred significant protection against both *S. typhimurium* hybrid challenge organisms. With hybrid H1 as the challenge organism, the protection resulting from *S. typhi* O antigen expression by the *E. coli* hybrid vaccinating strains was readily apparent when viewed in comparison with the absence of protection against H1 in the animals vaccinated with the *E. coli* parent strain F464. However, with H42 as the challenge organism, the influence of the *S. typhi* O antigens in those *E. coli* hybrid vaccinating strains was largely obscured by the nonspecific protective effect against hybrid H42. Vaccination with the Vi antigen-expressing (Vi-positive) *E. coli* hybrid WR3078 was seen to confer no protection advantage over vaccination with the Vi-nonexpressing (Vi-negative) *E. coli* hybrid F1061 against a challenge with either of the *S. typhimurium* hybrids. The best protection against both challenge organisms was conferred by vaccination with the live *S. typhi* strains Ty2 and the Vi-negative Ty2W. The level of protection conferred against H1 by these strains was about the same as that conferred against H42, and there was no essential difference between Ty2 and Ty2W in the protection conferred against either hybrid challenge organism.

DISCUSSION

The results of the present study indicate and reinforce several points that have been suggested or indicated in our earlier studies with this system. First, the immune response directed against the *S. typhi* O antigens expressed by an *S. typhimurium* hybrid challenge organism is important in protecting the animals against death (1). Second, this O antigen-specific protective effect is seen more clearly when a Vi-negative hybrid is employed as the challenge organism than when a Vi-positive hybrid is used (1, 2). Third, the presence of the Vi antigen in the vaccine or vaccinating strain does not contribute in any observable manner to the protection conferred against a Vi-positive *S. typhimurium* hybrid challenge organism (1, 3–5). In addition, the present findings show that the nonspecific protective effect that vaccination with *E. coli* F464 engenders against a challenge with *S. typhimurium* hybrid H42 (an effect that we believe is associated with Vi antigen expression by H42) does not occur when the Vi-negative *S. typhimurium* hybrid H1 is employed in this manner.

In our early studies with this system (1, 2; B. B. Diena, unpublished data), we noted that all *S. typhimurium* hybrids that had inherited and expressed the *S. typhi* Vi antigen-determining genes were, when used as challenge organisms, better protected against by most of the vaccines we tested, irrespective of the specificity of the vaccines for their surface antigens, than were *S. typhimurium* hybrids that had not inherited the Vi-determining genes. Because the inheritance of unlinked, unselected characters in the *S. typhi* × *S. typhimurium* mating system is very low (9), we assumed this nonspecific protective effect to be due to Vi antigen expression in these hybrids rather than to their inheritance of other relevant but undetected *S. typhi* charac-

ters, although the latter possibility has not been ruled out. In examining the virulence of *S. typhimurium* WR5004 and its *S. typhi* surface antigen-expressing hybrid derivatives administered intraperitoneally at low dose levels, it was observed that all of the Vi-positive hybrids were measurably slower (2 to 3 days) in bringing about the onset of deaths in nonimmunized mice than were *S. typhimurium* WR5004 and Vi-negative hybrids of this strain (1; B. B. Diena, unpublished data). We also noted in the present study, this 2- to 3-day delay in the onset of mouse deaths in the nonvaccinated control group challenged with *S. typhimurium* hybrid H42. Earlier (1), we speculated that this altered virulence pattern might be a factor in the protective effect of nonspecific vaccination against these Vi-positive hybrid challenge strains. At the present time, however, we are still not able to provide a fully satisfactory explanation for this effect. Nevertheless, we consider the protection conferred against *S. typhimurium* hybrid H42 by vaccination with *E. coli* F464, which expresses no surface antigens common to that hybrid, to be an improper response for a system intended to differentiate vaccines on the basis of their antigenic specificity and to reflect their potential as immunizing agents against typhoid fever in humans. The use of *S. typhimurium* hybrid H1 in the present comparison provides what we consider a more appropriate response, i.e., no significant protection is conferred by *E. coli* F464, and its hybrid derivatives are protective because of their expression of the *S. typhi* O antigens.

In previous studies with this system (1, 3–5), we have repeatedly observed the inability of vaccination with the Vi antigen to influence protection against *S. typhimurium* hybrid H42 or any other Vi-expressing *S. typhimurium* hybrid challenge organisms that we have examined. This has been the case regardless of whether the Vi antigen was administered to the animals intraperitoneally (1, 3–5) or orally (3, 5), in purified form (4, 5) or as a component of nonliving vaccinating organisms (1), or, as in the present study, as a component of live vaccinating strains (3). Inasmuch as *S. typhimurium* hybrid H42 is also seen to be less discriminating as a challenge organism than *S. typhimurium* hybrid H1, there seems to us no point in further employment of H42 for this purpose. Therefore, some consideration seems in order as to whether using Vi-negative *S. typhimurium* hybrid H1 as a challenge organism in the present mouse assay system can, in fact, provide a valid indication of the potential of a vaccine for preventing typhoid fever in humans.

We pointed out earlier (4) that our use of *S. typhimurium* hybrids expressing *S. typhi* antigens, as challenge organisms in the present system, has provided a different answer to the question of which *S. typhi* antigens are important in mouse protection than has the use of mucin-treated *S. typhi* as the challenge organism. In the latter system, which is currently employed as a laboratory assay for typhoid vaccines, the protective role of the Vi antigen has been well documented (11, 14, 19). However, neither the importance of the Vi antigen in mouse protection in that system nor its lack of involvement in protection in ours provides a convincing indication of the role of this antigen in human protection. As we have frequently stated, evidence for the role of any antigen in protection against typhoid fever in humans must come from studies conducted with human subjects. Accordingly, the desired assay system is one in which the laboratory test results are in agreement with the findings of human experience.

Although it has long been assumed by many workers that the Vi antigen is important in the protective immunization of humans against typhoid fever, there has never been, to our knowledge, any experimental evidence, either from field trials or volunteer studies, to support this belief. On the other hand, studies in recent years with the attenuated *S. typhi galE* mutant Ty21a (6, 7, 17, 18) have shown that effective human typhoid protection can be achieved by an immunization in which the Vi antigen is not involved at all. *S. typhi* Ty21a is Vi negative (7; our own observation). Nevertheless, when administered as a live, oral vaccine, Ty21a has been shown to be highly effective in protecting against typhoid fever, both in human volunteers (7) and in a recently concluded field trial that involved over 32,000 school children in Alexandria, Egypt (17, 18). Importantly, statistically significant protection is conferred by *S. typhi* Ty21a when the organism is grown under conditions in which its O antigens are synthesized, but not when it is grown under conditions in which its O antigens are not synthesized (7). Thus, in addition to showing that the Vi antigen is not required for protection, the behavior of this strain also indicates the importance of the *S. typhi* O antigens in human typhoid immunization.

At the present time, the only example we know of in which the Vi antigen can clearly be demonstrated to play a role in protective immunization is the standard mouse assay system for typhoid vaccines, in which *S. typhi*, an obligate human pathogen, is manipulated to serve, artificially, as a challenge organism. Although artificiality will always exist to some extent in any model system, our assay does utilize, we believe, a somewhat more natural infection model, in that the hybrid challenge strain is derived

from an organism that is actually a mouse pathogen. We think, therefore, that the absence of a detectable protective role for the Vi antigen in this system merits some consideration. This consideration is further warranted, we believe, by the continuing absence of any experimental evidence that might demonstrate a protective role for the Vi antigen in human typhoid immunity. If, as we suspect, the most effective vaccines for human typhoid immunization are seen to be live, avirulent, orally administered organisms such as *S. typhi* Ty21a, whose protective capability is dependent upon expression of the *S. typhi* O antigens, and not upon the Vi antigen, we believe that the use of *S. typhimurium* hybrid H1 as a challenge organism in the presently studied system will produce test results that are in line with those of human experience.

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A mouse-virulent Salmonella typhimurium hybrid, H42, which expresses the Salmonella typhi Vi antigen in addition to S. typhi O antigens 9 and 12, and a mouse-virulent S. typhimurium hybrid, H1, which expresses only the 9 and 12 antigens of S. typhi, were compared in their behavior as challenge organisms in a system developed to assay the protective capabilities of typhoid vaccines. Swiss-Webster white mice, vaccinated intraperitoneally with live E. coli hybrids hybrids expressing the S. typhi O antigens 9 and 12, were significantly

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protected against death from intraperitioneal challenge with each of the S. typhimurium hybrid strains. Vaccination with an E. coli hybrid expressing the S. typhi Vi antigen in addition to O antigens 9 and 12 was seen to confer no advantage in protection against either S. typhimurium hybrid challenge organism over that obtained by vaccination with an E. coli hybrid expressing only the O antigens of S. typhi. However, a notable difference in the behavior of the two S. typhimurium hybrids was seen in mice vaccinated with the parent of the E. coli hybrid vaccinating strains, E. coli F464, which expresses no surface antigens common to either of these S. typhimurium hybrid challenge organisms. A non-specific (with respect to the vaccinating strain) protective effect, believed to be associated with Vi antigen expression by the challenge organism, was seen against the challenge with S. typhimurium hybrid H42 after F464 vaccination, whereas no protection was conferred by F464 vaccination against the challenge with Vi non-expressing S. typhimurium hybrid H1. Inasmuch as neither S. typhimurium hybrid discriminates between the expression or non-expression of the Vi antigen in a vaccinating strain, it is concluded that the Vi non-expressing S. typhimurium hybrid H1, which more clearly indicates the vaccine-specific protective role of the S. typhi O antigens, and does not exhibit the non-specific protection response of hybrid H42, is the better choice as challenge organism for this vaccine assay system.